Description

Thus, the present invention relates to a combination product comprising at least one antisense oligonucleotide of the gene encoding MBD2 demethylase and at least one agent used in antitumor chemotherapy, for simultaneous, separate or prolonged use intended for the treatment of proliferative and inflammatory diseases.

In a particular embodiment, the antisense of the gene encoding MBD2 demethylase comprises at least 15 consecutive nucleotides of the sequence SEQ ID No.1 or of the sequence complementary thereto, or of SEQ ID No.2.

SEQ ID No.1 corresponds to the sequence described in GENEBANK under the accession number AF 072242 (Homo sapiens methyl-CpG binding protein MBD2 (MBD2) mRNA, complete cds).

SEQ ID No.1:

ggggcgtggccccgagaaggcggagacaagatggccgcccatagcgcttggaggacctaagaggcggtggccggg
gccacgccccgggcaggagggccgctctgtgcgcgcccgctctatgatgcttgcgcgcgtcccccgcgcgccgcgctgc
gggcggggcgggtctccgggattccaagggctcggttacggaagaagcgcagcgccggctggggaggggctggatg
cgcgcgcacccgggggggaggccgctgctgcccggagcaggaggaggggagagtgcggcgggcggcagcggcgct
ggcggcgactccgccatagagcaggggggccagggcagcgcgctcgccccgtccccggtgagcggcgtgcgcaggg
aaggcgctcggggcggcggccgtggccgggggcggtggaagcaggcgggccggggcggcggcgtctgtggccgtg
gccggggccggggccgtggccggggacggggacggggccggggccggggccgcggccgtcccccgagtggcggc
agcggccttggcggcgacggcggcggctgcggcggcggcggcagcggtggcggcggcgcccccccggcgggagccg
gtcccttcccgtcggggagcgcggggccggggcccaggggaccccgggccacggagagcgggaagaggatggattg
cccggccctcccccccggatggaagaaggaggaagtgatccgaaaatctgggctaagtgctggcaagagcgatgtctact
acttcagtccaagtggtaagaagttcagaagcaagcctcagttggcaaggtacctgggaaatactgttgatctcagcagttttg
acttcagaactggaaagatgatgcctagtaaattacagaagaacaaacagagactgcgaaacgatcctctcaatcaaaataa

gggtaaaccagacttgaatacaacattgccaattagacaaacagcatcaattttcaaacaaccggtaaccaaagtcacaaatc
atcctagtaataaagtgaaatcagacccacaacgaatgaatgaacagccacgtcagcttttctgggagaagaggctacaag
gacttagtgcatcagatgtaacagaacaaattataaaaaccatggaactacccaaaggtcttcaaggagttggtccaggtagc
aatgatgagacccttttatctgctgttgccagtgctttgcacacaagctctgcgccaatcacagggcaagtctccgctgctgt
ggaaaagaaccctgctgtttggcttaacacatctcaacccctctgcaaagcttttattgtcacagatgaagacatcaggaaaca
ggaagagcgagtacagcaagtacgcaagaaattggaagaagcactgatggcagacatcttgtcgcgagctgctgatacag
aagagatggatattgaaatggacagtggagatgaagcctaagaatatgatcaggtaactttcgaccgactttccccaagrgaa
aattcctagaaattgaacaaaaatgtttccactggcttttgcctgtaagaaaaaaaatgtacccgagcacatagagcttttaata
gcactaaccaatgccttttagatgtatttttgatgtatatatctattattcaaaaaatcatgtttatttgagtcctaggacttaaaatt
agtcttttgtaatatcaagcaggaccctaagatgaagctgagcttttgatgccaggtgcaatctactggaaatgtagcacttacg
taaaacatttgtttcccccacagttttaataagaacagatcaggaattctaaataaatttcccagttaaagattattgtgacttcact
gtatataaacatattttatactttattgaaaggggacacctgtacattcttccatcatcactgtaaagacaaataaatgattatattc
acaaaaaaaaaaaaaaaaaa

Among the preferred antisense sequences of the invention, more particularly noted is the sequence SEQ ID No.2, which corresponds to the complete messenger RNA of the demethylase in the antisense orientation:

cgcatgcatgcataagcttgctcgagtctagatttttttttttttttgtctgtgaatataatcatttatttgtctttacagtgatgatggaa
gaatgtacaggtgtccccttcaataaagtataaaaatatgtttatatacagtgaagtcacaataatctttaactgggaaatttattt
agaattcctgatctgttcttattaaaactgtgggggaaacaaatgttttacgtaagtgctacatttccagtagattgcacctggcat
caaaagctcagcttcatcttagggtcctgcttgatattacaaaagactaatttaagtcctaggactcaaaataaacatgatttttt
gaataatagatatatacatcaaaaatacatctaaaaaggcattggttagtgctattaaaaagctctatgtgctcgggtacatttttt
tcttacaggcaaaagccagtggaaacatttttgttcaatttctaggaattttcycttggggaaagtcggtcgaaagttacctgatc
atattcttaggcttcatctccactgtccatttcaatatccatctcttctgtatcagcagctcgcgacaagatgtctgccatcagtgct
tcttccaatttcttgcgtacttgctgtactcgctcttcctgtttcctgatgtcttcatctgtgacaataaaagctttgcagaggggttg
agatgtgttaagccaaacagcagggttcttttccacagcagcggagacttgccctgtgattggcgcagagcttgtgtgcaaag
cactggcaacagcagataaaagggtctcatcattgctacctggaccaactccttgaagacctttgggtagttccatggtttttat
aatttgttctgttacatctgatgcactaagtccttgtagcctcttctcccagaaaagctgacgtggctgttcattcattcgttgtggg
tctgatttcactttattactaggatgatttgtgactttggttaccggttgtttgaaaattgatgctgtttgtctaattggcaatgttgtatt
caagtctggtttacccttattttgattgagaggatcgtttcgcagtctctgtttgttcttctgtaatttactaggcatcatctttccagtt

ctgaagtcaaaactgctgagatcaacagtatttcccaggtaccttgccaactgaggcttgcttctgaacttcttaccacttggact
gaagtagtagacatcgctcttgccagcacttagcccagattttcggatcacttcctccttcttccatccgggggggagggccg
ggcaatccatcctcttcccgctctccgtggcccggggtcccctgggccccggccccgcgctccccgacgggaaagggac
cggctccgtcgacgcggcc

This antisense sequence was used in the context of the experiments presented in Example 1.

Thus, the invention is directed toward a combination product as mentioned above, in which the antisense comprises at least:

a) 15 consecutive nucleotides of the sequence SEQ ID No.1 or of the sequence complementary thereto, or of the sequence SEQ ID No.2, or

b) a sequence capable of hybridizing selectively with one of the sequences defined in a).

The expression "sequence capable of hybridizing selectively" is intended to mean the sequences which

hybridize with the abovementioned sequences at a level significantly greater than the background noise. The background noise may be related to the hybridization of other DNA sequences as are present, in particular other mRNAs that are present in the targeted tumor cells. The level of the signal generated by the interaction between the sequence capable of hybridizing selectively and the sequences defined by SEQ ID Nos. 1 and 2 above is generally 10 times, preferably 100 times, more intense than that of the interaction of the other DNA sequences generating the background noise. The level of interaction can be measured, for example, by labeling the sequence used as a probe with radioactive elements, such as ^{32}P. The selective hybridization is generally obtained by using very strict medium conditions (for example 0.03M NaCl and 0.03M sodium citrate at approximately 50°C-60°C). The hybridization can be carried out according to the usual methods of the state of the art (in particular Sambrook et al., 1989, Molecular Cloning : A Laboratory Manual).

The expression "agent used in antitumor chemotherapy" is intended to denote antineoplastic agents. Among these agents, mention may be made of:

- the compounds belonging to the bleomycin family (Mueller et al., Cancer, Vol. 40, p. 2787 (1977), Umezawa et al., Journal of Antibiotics, 19A, p. 210 (1966), US 4,472,304, FR2530639, and US 3,922,262), in particular bleomycin,
- the various cytolytic agents such as dacarbazine, hydroxycarbamide, asparaginase, mitoguazone and plicamycin,
- the methylating agents, such as streptozotocin (2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose), procarbazine (N-(1-methylethyl)-4-[(2-methylhydrazino)methyl]benzamide), dacarbazine

or DTIC (5-(3,3-dimethyl-1-triazenyl)-1H-imidazole-4-carboxamide), and temozolomide (8-carbamoyl-3-methylimidazo[5.1-d]-1,2,3,5-tetrazin-4-(3H)-one,

- the chloroethylating agents, such as HECNU (1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea), BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea or carmustine, Bristol-Meyers), ACNU (1-(2-chloroethyl)-3-(4-amino-2-methyl-5-pyrimidinyl)methyl-1-nitrosourea), CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea or lomustine), MeCCNU (1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea or semustine), fotemustine (1-[N-(2-chloroethyl)-N-nitrosoureido]ethylphosphonic acid diethyl ester) and clomesone (2-chloroethylmethylsulfonyl-methanesulfonate) (Pegg et al., Prog. Nucleic Acid Research Molec. Biol. 51: 167-223 (1995)). These agents are further described in Colvin and Chabner, Alkylating Agents. In: Cancer,
- other alkylating compounds such as agents of the type Ecteinascidin 743, and the duocarmycins (Boger et al. J. Org. Chem. 1990, 55, 4499; Boger et al. J. Am. Chem. Soc. 1990, 112, 8961; Boger et al. J. Am. Chem. Soc. 1991, 113, 6645; Boger et al. J. Am. Chem. Soc. 1993, 115, 9872; Boger et al. Bioorg. Med. Chem. Lett. 1992, 2, 759),

- the pro-apoptotic agents selected from glucocorticoid derivatives, topoisomerase inhibitors such as topoisomerase 2 inhibitors, for example anthracyclines, epipodophyllotoxin, such as etoposide, topoisomerase 1 inhibitors, for example camptothecin derivatives,

- the antimetabolites such as antifolates, for example methotrexate, antipurines, for example

6-mercaptopurine, antipyrimidines, for example 5-fluorouracil,

- from the antimitotics such as the vinca-alkaloids, taxoids such as taxotere.

These antineoplastic agents are described in Actualité Pharmaceutiques [Pharmaceutical News] No. 302 (Oct. 1992), pages 38 to 39, and 41 to 43, incoporated herein by reference.

In a preferred aspect, the invention is directed toward a combination product as defined above, in which the agent is selected from compounds belonging to the bleomycin family, in particular bleomycin.

In another particular embodiment, the invention relates to a combination product mentioned above, in which the antisense oligonucleotide of the gene encoding MBD2 demethylase is carried by a vector comprising a promoter which allows its effective expression in a eukaryotic cell. This vector may also comprise a poly A transcription termination sequence.

Preferably, the vector consists of a plasmid. In fact, the use of a plasmid is more economical and safer than the use of viruses. In addition, this embodiment of the invention allows readministration without triggering an immune response. This plasmid advantageously comprises a promoter, the antisense sequence according to the invention and a transcription terminating sequence. Preferably, the sequence of the antisense is inserted into the plasmid pcDNA3.1HisA from the company InVitrogen.

The product according to the invention may also comprise one or more pharmaceutically acceptable vehicle(s). It is

intended in particular for simultaneous, separate or prolonged use intended for the treatment of cancer.

In this sense, in a preferred embodiment, the formulations are suitable for administration by intratumor injection.

The techniques for transferring the plasmid into the target cells are well known to those skilled in the art. In particular, reference will be made to the techniques for electrotransfer into eukaryotic cells described in WO 99/01157 and Bettan et al., Bioelectrochemistry and Bioenergetics, 2000, 52:83-90. In WO 99/01157, a method for in vivo transfer of nucleic acids is proposed using weak electric fields between 1 and 600 V/cm. Other approaches are described in Wolf et al., Science 247, 1465-68, 1990; and Davis et al., Proc. Natl. Acad. Sci. USA 93, 7213-18, 1996), in which the DNA is associated with compounds intended to promote its transfection, such as proteins, liposomes, charged lipids or cationic polymers, such as polyethyleneimine, which are good in vitro transfecting agents (Behr et al., Proc. Natl. Acad. Sci. USA 86, 6982-6, 1989; Felgner et al., Proc. Natl. Acad. Sci. USA 84, 7413-7, 1987; Boussif et al., Proc. Natl. Acad. Sci. USA 92, 7297-301, 1995).

Thus, in accordance with the invention, the antisense can also be transferred in the form of double-stranded DNA or of a plasmid as mentioned above, possibly in combination with a molecule which promotes the transfer and/or using a weak electric field.

The invention also extends to any application for treating cancer, comprising the use of a combination product mentioned above and a third active substance used in the context of the treatment of the cancer. In this respect, the invention covers a tritherapy comprising the

administration of the combination product according to the invention and a third active substance.

Mbd2/demethylase is expressed in tumors in vivo and is overexpressed in a significant percentage of tumors in a manner similar to Dmnt1. Although our analysis of a limited number of tumors does not prove that Mbd2/demethylase is generally deregulated in cancer cells, our data are compatible with this model. Secondly, we show that the antisense-mediated inhibition of Mbd2/demethylase results in changes in genomic methylation and in an inhibition of tumorigenesis in vitro. Various methods of antisense expression have been used in order to exclude the possibility that the changes observed reflect a certain idiosyncratic property of the vector. Transient expression of the antisense is sufficient to inhibit the anchorage- and contact-inhibited growth, which indicates that Mbd2/demethylase is necessary for maintaining the transformed state, and that its inhibition has immediate effects on the growth of cancer cells.

Similarly, the introduction of a vector expressing the antisense of Mbd2/demethylase into human tumors, that had been passed in nude mice in the form of xenographs, resulted in a decrease in the growth of the tumor, which shows that Mbd2/demethylase is necessary for maintaining the transformed state. Whereas the expression of the Mbd2/demethylase antisense considerably inhibits tumorigenesis in vitro, it has a limited effect on tumors in vivo. This could reflect the difficulty that exists in effectively delivering and expressing the antisense vectors in all the cells of a tumor in vivo, rather than an indication of the limited impact of the inhibition of the target.

Since Mbd2/demethylase can either repress or demethylate

methylated genes, it is possible for a certain number of genes to be affected by one or other of these processes. Inhibition of the repression, mediated by Mbd2/demethylase, of the activity of methylated genes could result in an activation of a certain number of tumor suppressors. Moreover, the demethylase activity could be required for inhibiting an aberrant methylation of genes which are essential for the transformed phenotype. Inhibition of the demethylase could result in an ectopic methylation, essential genes being silenced stochastically.

Since the two activities of Mbd2/demethylase must affect a wide range of genes, a possible result could have been a collapse of the gene expression program. Such a possibility would have to have limited the therapeutic potential of the inhibition of Mbd2/demethylase. However, analysis of the gene scheme of the cells in which Mbd2/demethylase is inhibited does not support this hypothesis.

Thus, the inhibition of Mbd2/demethylase results in a repression and in an induction of the expression of the genes involved in the tumoral process, but does not present any disadvantage for a therapeutic application. Changes in gene expression after treatment with the Mbd2/demethylase antisense appear to be limited, however these changes, strengthened by an alkylating agent, are responsible for the strong inhibition of tumorigenesis in vitro.

Thus, the invention proposes the joint use of Mbd2/demethylase as an anticancer target, and a DNA alkylating agent. The fact that the cell cycle of normal cells is not affected by this treatment, and the fact that this treatment does not cause any massive changes in gene expression, increase the advantage of this target. The inhibition of Mbd2/demethylase could have a therapeutic effect on two levels, one in re-establishing the normal

state of genomic methylation by inhibition of a demethylase that is undergoing aberrant overregulation, and another in preventing that which causes incorrectly methylated tumor suppressor genes to become silent, which genes are essential to maintaining an appropriate regulation of cell growth.

Example 1: Combination of gene therapy (intratumor electrotransfer of plasmids encoding the DNA demethylase antisense) and of chemotherapy (intramuscular injection of bleomycin)

Two series of experiments were carried out in nude mice weighing 18 to 20 g. The mice were implanted on one side with H1299 tumor grafts (human non-small cell lung tumors) of approximately 20 mm^3. The tumors developed, to reach a volume of 20 to 150 mm^3. The mice were sorted as a function of the size of the tumors and were divided up into homogeneous batches reaching tumor volumes of 50 to 80 mm^3 (n=10 to 13). The mice were anesthetized with a mixture of ketamine and xylazine.

1.1 Experiment 1: Effect on tumor growth

The results are illustrated in figure 1 and the statistical analysis is given in table 1 below.

TABLE 1

STATISTICAL ANALYSIS

Experiment 1

	Day 1000 mm^3 (median) #
Group 1: untreated tumors	14.50
Group 3: 25 µg bleomycin	44.40
Group 4: DNA demethylase	29.10

antisense Group 6: DNA demethylase antisense + 25 µg bleomycin	52.01			
	Student's t test	Log-Rank		
Statistical comparison	Mean comparison	Kaplan-Meier Risk of reaching 1000 mm^3 of tumor volume		
DNA demethylase antisense versus untreated	p<0.0001	***	p<0.0001	***
25 µg bleomycin versus untreated	p<0.0001	***	p<0.0001	***
DNA demethylase antisense + 25 µg bleomycin versus 25 µg bleomycin	p=0.1079	NS	p=0.1946	NS
DNA demethylase antisense + 25 µg bleomycin versus untreated	p<0.0001	***	p<0.0001	***

1.1.1 Control tumors:

A series of tumors was subjected to no treatment.

1.1.2 Tumors treated with the gene encoding the DNA demethylase antisense, alone:

Five electrotransfers of 50 µg of plasmid in 80 µl of 150 mM NaCl were carried out in the tumors on the days indicated by the arrows. The plasmid solution was injected longitudinally at the periphery of the tumor using a Hamilton syringe. The lateral faces of the tumors were coated with conducting gel and the tumors were placed between 2 flat stainless steel electrodes 0.4 to 0.7 cm apart. Twenty to 30 seconds after the injection, the plasmids were electrotransferred using a commercial

(square) electrical pulse generator (Jouan Electropulser PS 15). Each tumor was subjected to 500 V/cm delivered in 8 pulses lasting 20 msec at a frequency of 1 Hertz.

1.1.3 Tumors treated with bleomycin alone:

Twenty-five µg of bleomycin/animal in 50 µl of 150 mM NaCl were injected bilaterally into the tibialis cranialis muscle and, 30 minutes later, each tumor was subjected to 1 electrotransfer as explained above.

1.1.4 Tumors treated with a combination of the 2 treatments (antisense and bleomycin):

Twenty-five µg of bleomycin/animal in 50 µl of 150 mM NaCl were injected bilaterally into the tibialis cranialis muscle and, 30 minutes later, 50 µg of antisense plasmid in 80 µl of 150 mM NaCl were injected and electrotransferred. Four other electrotransfers of 50 µg of antisense plasmid in 80 µl of 150 mM NaCl were subsequently carried out in the tumors on the days indicated by the arrows.

The tumor volumes were measured individually for each tumor using an electronic slide gauge with a digital display, according to the formula (length × width × thickness)/2.

The median of the tumor volumes was reported in the form of a graph, as a function of time.

1.2 Experiment 2: Effect on tumor growth

The results are illustrated in figure 2 and the statistical analysis is given in table 2 below.

TABLE 2

STATISTICAL ANALYSIS

Experiment 2

	D 1000 mm^3 (median) #
Group 1: NaCl/ET	20.90
Group 2: 25 µg bleomycin	38.00
Group 3: DNA demethylase antisense	38.60
Group 4: DNA demethylase antisense + 25 µg bleomycin	52.00

Statistical comparison	Student's t test Mean comparison	Log-Rank Kaplan-Meier Risk of reaching 1000 mm^3 of tumor volume		
DNA demethylase antisense versus NaCl/ET	p=0.0201	*	p=0.0029	**
25 µg bleomycin versus NaCl/ET	p=0.0008	***	p=0.0001	***
DNA demethylase antisense + 25 µg bleomycin versus 25 µg bleomycin	p=0.0088	**	p=0.0056	**
DNA demethylase antisense + 25 µg bleomycin/NaCl/ET	p=0.0001	***	p<0.0001	***

#: number of days to reach 1000 mm^3 of tumor volume

1.2.1 Control tumors:

Five electrotransfers of 80 µl of 150 mM NaCl were carried out in the tumors on the days indicated by the arrows.

1.2.2 Tumors treated with the gene encoding the DNA demethylase antisense, alone

Fifty µl of 150 mM NaCl were injected bilaterally into the tibialis cranialis muscle and, 30 minutes later,

an electrotransfer of 50 µg of antisense plasmid in 80 µl of 150 mM NaCl was carried out. Four other electrotransfers of 50 µg of antisense plasmid in 80 µl of 150 mM NaCl were subsequently carried out in the tumors on the days indicated by the arrows.

1.2.3 Tumors treated with bleomycin alone:

Twenty-five µg of bleomycin/animal in 50 µl of 150 mM NaCl were injected bilaterally into the tibialis cranialis muscle and, 30 minutes later, each tumor was injected with 80 µl of 150 mM NaCl and subjected to an electrotransfer. Four other electrotransfers of 80 µl of 150 mM NaCl were subsequently carried out in the tumors on the days indicated by the arrows.

1.2.4 Tumors treated with a combination of the 2 treatments (antisense and bleomycin):

Twenty-five µg of bleomycin/animal in 50 µl of 150 mM NaCl were injected bilaterally in the tibialis cranialis muscle and, 30 minutes later, an electrotransfer of 50 µg of antisense plasmid in 80 µl of 150 mM NaCl was carried out. Four other electrotransfers of 50 µg of antisense plasmid in 80 µl of 150 mM NaCl were subsequently carried out in the tumors on the days indicated by the arrows.

The tumor volumes were measured individually for each tumor using an electronic slide gauge with a digital display, according to the formula (length × width × thickness)/2.

The median of the tumor volumes was reported in the form of a graph, as a function of time.

1.3 Results and conclusion

The combination of gene therapy with the gene encoding the human DNA demethylase antisense and of chemotherapy with bleomycin makes it possible to induce a cumulative delay of 31 to 38 days in the growth of H1299 tumors.

Such a delay in tumor growth was never achieved with the treatments administered separately, such as the gene therapy alone (15 to 18 days) or the chemotherapy alone (17 to 30 days) (table 3 below).

TABLE 3

Combination of gene therapy and of chemotherapy

Effect of multiple intratumor electrotransfers of plasmids encoding the human DNA demethylase antisense, combined with a treatment with bleomycin, on the growth of H1299 tumors

a) Delay in tumor growth

	Experiment 1		Experiment 2	
	D1000 *	Delay in growth Treatment versus untreated	D1000 *	Delay in growth Treatment versus electro/NaCl
Untreated ET/NaCl	D14		D21	
Demethylase antisense	D29	15 days	D39	18 days
25 µg bleomycin	D44	30 days	D38	17 days
Demethylase antisense/25 µg bleomycin	D52	38 days	D52	31 days

D1000* = number of days required to reach a tumor volume of 1000 mm^3

The combination of the gene therapy and the chemotherapy induces a synergistic effect on the tumor cure rate, since a tumor cure rate of 30 to 40% was obtained with the combined treatment, compared with 10% only with the treatments administered separately (table 4 below).

TABLE 4

Combination of gene therapy and of chemotherapy

b) Tumor cure rate

	Experiment 1	Experiment 2
	Number of tumors cured	Number of tumors cured
Untreated NaCl/electro	0/11	 011
Demethylase antisense	0/13	1/10 D53
25 µg bleomycin	1/13 D54	1/11 D53
Demethylase antisense/25 µg bleomycin	3/11 D33/D69/D69	4/10 D32/D35/D53/D53

Rem: the tumors cured are tumors which are no longer measurable

Dx: absence of tumors up to the day indicated, beyond which the mouse died

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